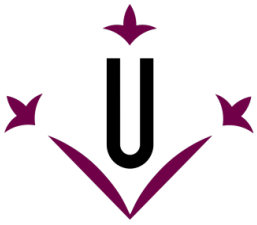

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Interference of plant volatiles on pheromone receptor neurons of male *Grapholita molesta* (Lepidoptera: Tortricidae)

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Abstract

In moths, sex pheromone components are detected by pheromone-specific olfactory receptor neurons (ph-ORNs) housed in sensilla trichodea in the male antennae. In *Grapholita molesta,* ph-ORNs are highly sensitive and specific to the individual sex pheromone components, and thus help in the detection and discrimination of the unique conspecific pheromone blend. Plant odours interspersed with a sub-optimal pheromone dose are reported to increase male moth attraction. To determine if the behavioural synergism of pheromone and plant odours starts at the ph-ORN level, single sensillum recordings were performed on *Z*8-12:Ac and *E*8-12:Ac ph-ORNs (*Z*-ORNs and *E*-ORNs, respectively) stimulated with pheromone-plant volatile mixtures. First, biologically meaningful plant-volatile doses were determined by recording the response of plant-specific ORNs housed in sensilla auricillica and trichodea to several plant odorants. This exploration provided a first glance at plant ORNs in this species. Then, using these plant volatile doses, we found that the spontaneous activity of ph-ORNs was not affected by the stimulation with plant volatiles, but that a binary mixture of sex pheromone and plant odorants resulted in a small (about 15 %), dose-independent, but statistically significant, reduction in the spike frequency of *Z*-ORNs with respect to stimulation with *Z*8-12:Ac alone. The response of *E*-ORNs to a combination of *E*8-12:Ac and plant volatiles was not different from *E*8-12:Ac alone. We argue that the small inhibition of *Z*-ORNs caused by physiologically realistic plant volatile doses is probably not fully responsible for the observed behavioural synergism of pheromone and plant odors.

Key words: Single sensillum recording, olfactory receptor neuron, plant volatiles, sex pheromone

1. Introduction

Semiochemicals play an important role in insect communication (Bruce and Pickett, 2011; Beyaert and Hilker, 2014). Male moths follow the pheromone plume trails emitted by conspecific females for mating (McNeil, 1991; Landolt and Phillips, 1997). Moreover, male and female moths are attracted to host plant volatiles (Bruce and Pickett, 2011) derived from a large variety of secondary metabolites (Pichersky and Gershenzon, 2002). In addition to pheromone cues, males also use host plant cues to find females to mate, since females choose suitable host plants to lay eggs (Landolt and Phillips, 1997). For successful mate and host location it is crucial to detect the right proportion of individual components (i.e., odorants) in the volatile blend (i.e., odour) (Bruce and Pickett, 2011; Baker et al., 2012). The simultaneous presence of pheromone and plant odours could either help locating a mate, mask the female pheromone, or be neutral, without any effect on the female emitted pheromone (Deisig et al., 2014). There is evidence that the behavioural response of males to sex pheromone is increased by host plant volatiles (Reddy and Guerrero, 2004). Currently, efforts are dedicated to investigate the potential use of pheromones and other semiochemicals in pest management (Szendrei and Rodriguez-Saona, 2010; Witzgall et al., 2010).

In the last decade several studies have aimed to understand how the mixture of pheromone and plant odorants is reported by olfactory receptor neurons (ORNs) to higher processing centers in the brain, such as the antennal lobe (AL) (De Bruyne and Baker, 2008; Deisig et al., 2014). In moths, pheromone components are detected by highly specialised ORNs housed in sensilla trichodea, and all

pheromone-specific neurons converge in the macroglomerular complex (MGC) of the AL (Hansson and Anton, 2000; Baker et al., 2012). Both generalist and specialist ORNs housed in different sensilla types are involved in the detection of general odorants, including plant volatiles (Andersson et al., 1995; 1996; Ansebo et al., 2005, Deisig et al., 2012; Binyameen et al., 2012), and converge in many ordinary glomeruli (OG) in the AL (Hillier and Vickers, 2007; Deisig et al., 2014). Integration of pheromone and plant odours takes place in the AL, however there is evidence that odours also interact at the peripheral receptor level in pheromone-specific ORNs. For example, in *Helicoverpa (=Heliothis) zea* (Boddie), stimulation with binary mixtures of the major pheromone component, (*Z*)-11-hexadenal, and increasing dosages of either linalool or (*Z)*-3-hexenyl acetate, significantly synergise ph-ORNs firing rate compared with responses to the major pheromone component alone (Ochieng et al., 2002). By contrast, electrophysiological studies on ph-ORNs of *Heliothis virescens* (Fabricius) (Hillier and Vickers, 2011), *Spodoptera littoralis* (Boisduval) (Party et al., 2009) and *Agrotis ipsilon* (Hufnagel) (Deisig et al., 2012) have found that firing activity to pheromone is suppressed when plant odorants are co-applied. So, both excitation and inhibition to mixtures of pheromone and plant stimuli are observed at the peripheral level.

The oriental fruit moth, *Grapholita molesta* (Busck), is an oligophagous pest of stone and pome fruits. Larvae bore to new growth shoots and cause economic damage (Rothschild and Vickers, 1991). Female *G. molesta* emit a three-component blend of (*Z*)-8 dodecenyl acetate (*Z*8-12:Ac), (*E*)-8 dodecenyl acetate (*E*8-12:Ac), and (*Z*)-8 dodecenyl alcohol (*Z*8-12:OH), at a ratio of 100:6:10, respectively. A synthetic mixture of the blend is used in pest management (Roelofs et al., 1969; Linn

and Roelofs, 1983; Kong et al., 2014). Field studies report that male and female *G. molesta* are attracted to host-plant volatile blends (Il'ichev et al., 2009; Lu et al., 2012, 2014), and that terpinyl acetate (Knight et al., 2014) and (*Z*)-3 hexenyl acetate (Yu et al., 2014) increase male captures in pheromone traps. A 5-component plant odour blend behaviourally attractive to female *G. molesta* (Piñero and Dorn, 2007) synergises male response to a sub-optimal pheromone dose in the wind tunnel (Varela et al., 2011a), and addition of citral to *Z*8-12:Ac increases electroantennogram (EAG) responses compared to *Z*8-12:Ac alone (Faraone et al., 2013).

In this study, we explore whether physiological changes at the peripheral receptor level could explain the behavioural synergism produced by the mixture of pheromone and plant odors in male *G. molesta* (Varela et al., 2011a). We made single sensillum recordings from *Z*8-12:Ac and *E*8-12:Ac specific-ORNs (Ammagarahalli and Gemeno, 2014) stimulated with sex pheromone and plant volatiles independently or mixed in a blend, to determine if the response of these ORNs to sex pheromone is altered by co-stimulation with plant volatiles. We tested three plant blends with reported behavioural activity (Piñero and Dorn, 2007; Il'ichev et al., 2009; Lu et al., 2012), individual components from each blend, and additional odorants that could be biologically relevant. We tested them at several doses to account for possible concentration effects. Plant volatiles were tested first in non-pheromone ORNs from sensilla auricillica and trichodea to characterize the response of these yet unexplored ORNs, and to determine biologically-relevant plant doses to be used in the pheromone-plant interaction test.

2. Materials and methods

2.1. Insects

The colony of *G. molesta* was established at the University of Lleida, Spain, in 2005 with individuals formerly collected in peach orchards and reared in laboratory in Piacenza, Italy. Larvae were reared on a semi-synthetic diet modified from Ivaldi-Sender (1974) under a L16:D8 photoperiod at 25 ± 1 ^{o}C. Male pupae were placed in 4-l polypropylene containers provided with a cotton ball soaked in 10 % sucrose solution. Adult emergence was checked daily and adults were used when 2-4 days old. Care was taken not to expose adults to synthetic odours before the tests.

2.2. Odorant stimuli

Individual volatile compounds (i.e., odorants) and their blends (i.e., odours), were tested in the study (Table 1, chemical details in Table S1). The "Chinese" plant blend was identified from pear fruit volatile collections and it attracts males and females in the field and in the laboratory (Lu et al., 2012). The "Swiss" blend was identified from peach shoot volatiles and it attracts mated females in the laboratory (Piñero and Dorn, 2007) and synergizes male response to a suboptimal pheromone dose in the laboratory (Varela et al., 2011a). Finally, the "Australian" blend, which was identified from peach shoot volatiles, but has a different composition than the Swiss blend, attracts males in the field (Il'ichev et al., 2009). We prepared three plant blends emulating the "Australian", "Chinese", and "Swiss" blends (Table 1). Selected compounds from these blends, and others that have shown behavioural or

electrophysiological activity in *G. molesta* (Faraone et al., 2013; Knight et al., 2014), or that are released by peach or apple plants (Natale et al., 2003, Casado et al., 2006, Wang et al., 2009, Lu et al., 2012), were tested individually (Table 1).

Sex pheromone compounds were provided by Pherobank (The Netherlands) with an initial purity ≥99 %. Gas chromatographic analysis revealed that *Z*8-12:Ac contained 0.38 % *E*8-12:Ac, and that *E*8-12:Ac contained 0.24 % *Z*8-12:Ac. Pure pheromone and plant compounds were weighed and diluted in *n*-hexane to prepare 10 *µ*g/µl stock solutions. The pheromone blend consists of a mixture of *Z*8-12:Ac, *E*8-12:Ac and *Z*8-12:OH in a 100:6:10 ratio.

2.3. Electrophysiological recordings

Males were immobilized using CO_2 for 10 s and were mounted on a handcrafted poly(methyl methacrylate) insect holder. The insect was inserted through a vertical hole drilled in the holder´s body and the protruding head was restrained by fixing a piece of adhesive cloth tape between the head and the holder`s surface. The antennae were carefully laid on a slant surface attached to the holder´s top that was lined with double sided sticky tape, and were oriented for easy access with the electrodes. To record from sensilla located on the scaled area (sensilla trichodea or auricillica), scales were removed by gently rolling the antennae on the sticky tape, and the remaining scales were removed individually with the help of a tungsten electrode. Sub-millimetric smoking paper strips placed over the antennae and glued on the sticky surface prevented antennal torsion. A stereo microscope (objective 2x, oculars 25x, zoom range 0.8-12.5, Leica Microsystems,

Madrid, Spain) was used in performing these operations and to visualize the recordings. These were obtained by means of electrolytically (saturated KNO_2) sharpened tungsten microelectrodes (0.125-mm diameter, 99.98 % purity, Advent Research Materials Ltd, England). The reference electrode was inserted in the head through the mouth parts. The recording electrode was situated near the base of a randomly chosen sensillum and pushed gently inward with the help of a manual micromanipulator (NMN-25, Narishige, Japan) until spikes were detected. Flagellomeres 10-35 were sampled. Recordings from sensilla auricillica were made in the distal scaled area, and those from sensilla trichodea were distributed randomly in the scaled and scale-free areas. The signal from the recording electrode was pre-amplified (10x gain, Universal Single Ended Probe, Syntech, Germany), filtered (1 KHz and 300 Hz for high and low cut-off filters, respectively), digitized (IDAC-4, Syntech, Germany), and analyzed in a PC (AutoSpike v.3.9, Syntech, Germany). Sampling rate of the recording wave signal was 10666.7 samples s^{-1}. The setup was mounted on an anti-vibration table (63-511, TMC Ametek, USA) and was shielded by a Faraday cage to reduce low frequency noise.

2.4. Odour stimulation

Dilutions were applied as 1 μl aliquots (1 μl micropipettes, Drummond Scientific Co., USA) on 1 x 20 mm *n*-hexane-pre-cleaned filter paper strips (#1, Whatman International Ltd, England). After having dried (5 min) the filter papers were introduced in *n*-hexane-pre-cleaned 100 μl glass micropipettes (1.2 mm internal diameter, Blaubrand® Intramark, Germany) which were then stored in glass test tubes sealed with PTFE-coated screw caps until used. New stimuli cartridges were

prepared each day, and a given stimulus cartridge was not used for more than 10 stimulations. Air flow was generated by two diaphragm aquarium pumps connected to a 3-way solenoid valve (CS-55, Syntech, Germany). A 0.5-l/min flow of charcoal-filtered and humidified air blew continuously over the insect preparation through a 5-mm internal diameter plastic tube placed 15-20 mm from the preparation (air velocity at exit = 0.4 m/s). A stimulus cartridge was attached to the puff-flow with the side bearing the filter paper positioned 0.4 cm down from the recording point and perpendicular to the direction of the continuous air flow. A 0.2-l/m charcoal-filtered room air flow was puffed through the odour cartridge towards the recording spot for 200 ms (air velocity at exit = 2.9 m/s). The flow of continuous humid air was decreased by 0.2-l/min during the puff. Time interval between puffs was at least 60 s, but longer if needed to let the spike activity return to pre-stimulation levels. A maximum of 4 sensilla were recorded per insect, and at least a 30 min interval between two sensilla recordings was allowed. The air around the preparation was constantly renewed with an exhaust to minimize contamination. Test tubes for keeping stimulus pipettes were rinsed with acetone and heated at 250 ^{o}C overnight before being reused.

2.5. Response of non-pheromone specific ORNs to plant odorants

In order to choose biologically relevant plant doses for testing the response of pheromone-specific ORNs to pheromone and plant volatiles, we first determined the response of ORNs housed in sensilla auricillica, and of non-pheromone ORNs housed in sensilla trichodea, both of which are the most likely receptor neurons of plant volatiles (Ansebo et al., 2005; Binyameen et al., 2012), to several plant

volatiles at several doses. In *G. molesta* most of the sensilla trichodea (64 %) house one ORN responding only to *Z*8-12:Ac (*Z*-ORNs), 7 % house one ORN responding strongly to *E*8-12:Ac (*E*-ORNs) and weakly to *Z*8-12:Ac, and 29 % have ORNs that do not respond to the pheromone compounds (Ammagarahalli and Gemeno, 2014). Sensilla trichodea distribute evenly throughout the flagellum´s surface, whereas sensilla auricillica, which are readily distinguishable for their flattened shape and small size, occur mainly on the distal edge of the flagellomere and are more abundant in the scaled area (Ammagarahalli and Gemeno, 2014). Olfactory neurons in sensilla trichodea were first stimulated with individual sex pheromone compounds (1 ng of each, *Z*8-12:Ac, *E*8-12:Ac, *Z*8-12:OH) to determine if they were pheromone-specialist. Non pheromone-specific ORNs were further stimulated with 0.01 μg of the pheromone blend and 0.1 μg of several biologically relevant plant odorants, randomising the order of stimuli among ORNs. Preliminary tests showed that sensilla auricillica do not house pheromone specific ORNs, so they were stimulated with individual plant odorants (0.1 μg), with each of the 3 individual pheromone components (0.01 μg) and with the pheromone blend (0.01 μg). The order of stimuli was *n*-hexane, followed by the pheromone blend and its components, and the plant odorants in random order. ORNs from both sensilla types with relatively strong responses to a given plant compound were further stimulated with increasing doses (0.001 to 10 μg) of the most sensitive compounds to obtain dose-response curves.

2.6. Response of pheromone-specific ORNs to pheromone and plant odorants

Experiments were carried out to test whether individual plant compounds or plant blends (Swiss, Chinese, and Australian) (Table 1), affect the response of

pheromone-specific ORNs to their sex pheromone ligands. Dose-response curves from plant-ORNs showed that a physiologically-relevant range of plant odorant doses was 10 to 100 ng. A fixed concentration of sex pheromone (*Z*8-12:Ac or *E*8-12:Ac, 0.1 ng) was mixed with increasing concentrations of plant volatiles to make 1:0, 1:1, 1:10, 1:100 and 1:1000 pheromone:plant blends. Plant blends were tested in *Z*- and *E*-ORNs but plant odorants were not tested in *E*-ORNs due to their sparse number and distribution. Solvent (1 *µ*l on filter paper) and individual plant volatiles or plant blends (100 ng) were control treatments. To make the pheromone:plant blends, 100 µl of a 1 ng/*µ*l pheromone stock solution (*Z*8-12:Ac or *E*8-12:Ac) was added to a 2 ml vial, and the volume was completed to 1 ml by adding *n*-hexane and different volumes of 1 ng/*µ*l, 100 ng/*µ*l and 10 µg/*µ*l plant volatile solutions. Pheromone and pheromone plus plant solutions were prepared on the same day using the same pheromone stock solution, and therefore all of them contained the same pheromone concentration.

ORNs were first stimulated with 0.1 ng of *Z*- or *E*8-12:Ac to determine their ligand specificity. Once pheromone specificity was determined, the ph-ORNs were stimulated with the same treatment order: hexane, sex pheromone ligand (*Z*8-12:Ac or *E*8-12:Ac, 0.1 ng), plant volatiles (odorants or blends, 100 ng), pheromone:plant blends with a constant pheromone dose (0.1 ng) and increasing doses of the plant volatile (1:1, 1:10, 1:100 and 1:1000), and a second 0.1 ng *Z*- or *E*8-12:Ac puff at the end of the treatment run to control for possible neuron adaptation.

2.7 Spike and statistical analyses

When more than one spike size was detected, they were sorted by their shape and amplitude. ORNs were labelled large, medium and small (L, M and S, respectively) according to their relative size in each sensillum. For each puff, the number of spikes during a 1-s pre-stimulation period was subtracted from the number of spikes during a 1-s post-stimulation period to obtain the relative number of spikes per second, and this variable was analysed statistically. To determine if plant volatiles affect the response of pheromone-specific ORNs to sex pheromone, we used a model in which the difference in spikes before and after the puff was a function of the pheromone:plant dose and the plant volatile composition. The effect of plant blends on *Z*- and *E*-ORNs was analysed separately. Since the data were not normally distributed we run generalized linear models (GLM) in R (Bolker et al., 2009; R Core Team, 2013). Multiple pair-wise comparisons among treatment means were performed with least-squares means method using the "lsmeans" and "mcp" packages of R (R Core Team, 2013). Raw data, R codes and R outputs are available as supplementary material.

3. Results

3.1. Response of non-pheromone specific ORNs to plant odorants

Both excitation and inhibition were observed, but in general, responses were very similar to solvent stimulation, and very few ORNs showed any specialization. The number of ORNs per sensilla varied from one to three, with distinguishable large, medium, and small spike amplitudes. In most cases, only one neuron per sensillum was clearly excited by the plant odorants.

3.1.1. Sensilla trichodea

Sixty-four ORNs housed in 25 sensilla trichodea of 12 individuals were tested with 3 to 10 plant odorants, sex pheromone and *n*-hexane. Most of the sensilla (66 %) housed 3 ORNs (large, medium and small amplitude), whereas the remaining 34 % housed 2 ORNs (large and small amplitude). The spontaneous activity of large amplitude ORNs was <5 spikes s^{-1}, whereas that of medium and small amplitude ORNs was between 19 and 33 spikes s^{-1} (Table S2). Stimulation with plant odorants elicited between -36 and 79 spikes s^{-1} (Fig. 1A, ORNs 14S and 8S, respectively). Most ORN responses to plant volatiles were very similar to the response elicited by *n*-hexane (HEX, yellow colour range in raster plot, Fig. 1A). Specific response to one or a few plant odorants was rare, but a group of four small-amplitude ORNs (6 % of all the ORNs) produced relatively high spike counts to stimulation with farnesene (FAR) (Fig. 1A, ORNs 3S, 8S, 9S, 18S), while they showed strong inhibition to the other compounds (Fig. 1A, ORNs 8S, 9S). Spontaneous activity of neurons co-

localized in the same sensilla as the FAR-specific ORNs was practically unchanged by plant odorant stimulation (Fig. 1A, ORNs 3L, 3M, 8L, 9L, 18L). The spiking response of FAR-ORN 8S is illustrated in Fig. 2A. Few other ORNs in sensilla trichodea showed some degree of specialization. Benzaldehyde (BZA) inhibited ORN 14S and excited ORN 12S, pear ester (PE) excited ORN 4S, terpinyl acetate (TA) excited ORN 15S, and (*Z*)-3-hexenyl acetate (Z3HA) excited ORN 10S. The 6 ORNs housed in sensilla trichodea 24 and 25, were stimulated with 10 additional compounds but did not show specificity (data not shown). Only one ORN (6S) showed some response to sex pheromone (PHE).

Dose-response curves of sensilla trichodea ORNs were only made for three of the four FAR-specific neurons. All of them showed a typical sigmoidal-shape response in the log-dose scale, with little excitation to 1 and 10 ng doses and a sharp increase in the response to doses from 10 ng to 10 µg (Fig. 3A, Fig. 2A). *n*-hexane stimulation produced minute changes in spontaneous activity (Fig. 1A, Fig. 3A).

3.1.2. Sensilla auricillica

Eighty eight ORNs from 40 sensilla auricillica from 20 males were tested with 5 to 10 plant odorants, sex pheromone and *n*-hexane (Fig. 1B). Another 60 ORNs from 20 sensilla from 8 males were tested with 18-20 plant odorants, the pheromone blend and its individual components and *n*-hexane (Fig. 1B). More than half (67 %) of all the sensilla auricillica housed 3 ORNs, whereas a smaller percentage (29 %) housed two neurons, and the remaining 3 % sensilla housed a single neuron. The

spontaneous activity of large spike-amplitude ORNs was between 1 and 2 spikes s^{-1} in sensilla housing 2 or 3 neurons, but it rose to 26 spikes s^{-1} in sensilla housing just one ORN (Table S2). Medium and small spike-amplitude ORNs had between 24 and 34 spikes s^{-1} (Table S2). Fig. 2B shows the response of an auricillic sensillum (52 in Fig. 1B) whose large ORN responded to methyl salicylate (MS), the medium one to 1-octen-3-ol (3OH), whereas the small one was silent.

Stimulation with plant odorants elicited between -35 (ORN 53L) and 97 (ORN 4L) spikes s^{-1}, but most ORN responses were very similar to the response elicited by *n*-hexane (yellow range colour, Fig. 1B), and very little specialization was observed. Some ORNs showed moderate to high excitation to most of the plant odorants tested, including pheromone (2S, 3S, 5L, 14S, 19L, 21M, 31L, 33S, 36S, 37M, 41M, and 58M), but these were cells that normally showed relatively high responses to *n*-hexane. A few ORNs were broadly inhibited (9L, 23S and 32M) (Fig. 1B). Strong excitation and inhibition to different compounds for the same ORN was observed in several ORNs (e.g., 1L, 4L, and 53L). Out of the 60 ORNs stimulated with 18-20 plant odorants, two of them (52L, 53L) were relatively specific to methyl salicylate (MS) whereas a third one (52M) showed specificity to 1-octen-3-ol (3OH). ORN 32M was inhibited by most compounds but excited by benzonitrile (BZN), and cell 22S was excited by the pear ester (PE).

Dose-response curves were obtained for 5 plant odorants on 9 different ORNs (Fig. 3B-F). Except for the benzaldehyde ORN (Fig. 3F), for the rest of the plant odorants there was a typical dose-response pattern, where at low doses the ORNs were little excited, but at higher doses they were very responsive. Some ORNs (BZN 3S, and TA 37M and 37S, Fig. 3B and C) were not affected by the increase in plant odorant dose, but these cells were not very specific to these compounds (Fig. 1B).

The level of response of the auricillic ORNs was similar to that of the trichodea FAR ORN, with the 100 ng dose producing about 50 spikes s^{-1}. *n*-hexane stimulation produced minute changes in spontaneous activity in ORNs from sensilla auricillica (Fig. 1B, Fig. 3).

3.2. Response of pheromone-specific ORNs to mixtures of pheromone components and plant volatiles

Recordings were obtained from 112 *Z*-ORNs and 15 *E*-ORNs from 34 and 7 male moths, respectively. The percentage of sensilla with one, two, or three neurons was 60 %, 31 % and 9 %, respectively in *Z*-ORNs, and 73 %, 27 % and 0 %, respectively in *E*-ORNs. ORNs co-localized with pheromone ORNs did not respond to stimulation with sex pheromone or plant volatiles. *Z*- and *E*-ORNs produced less than 3 spikes s^{-1} upon stimulation with plant volatiles or *n*-hexane (Fig. 4B, and Fig. 5B and C), however they were clearly excited (about 40 spikes s^{-1}) by a medium dose (0.1 ng, Ammagarahalli and Gemeno, 2014) of their respective pheromone ligands (Fig. 4A and 5A).

Although Z-ORNs did not respond to plant stimuli alone, the addition of plant *odorants* or plant *blends* to the sex pheromone resulted in a relatively small (about 15 %) but statistically significant reduction in the response of *Z*-ORNs to *Z*8-12:Ac (Figs. 4 and 5, A and B) ($df = 7$, $F = 316$, $P<0.001$, for plant odorants; $df = 7$, $F = 88$, $P<0.001$ for plant blends). The reduction of *Z*-ORN response to pheromone was independent of the amount of plant odorant present in the mix, *i.e.,* all the plant odorant doses reduced the response and higher plant volatile doses did not result in further reduction (Fig. 4B). The reduction of plant blends on *Z*-ORN pheromone

response was only at the 1:1 and 1:1000 pheromone:plant ratios, and only with respect to the second pheromone puff, with no clear dose-response trend (Fig. 5B). Odorants differed in the reduction that they elicited in Z-ORNs (df=10, F=6.94, P<0.001) with BZA and TA producing less spikes than BZN, CIT and EB (Fig. 4C). When analysed individually, only 4 plant odorants decreased responses to the first pheromone puff, and they did so at the pheromone:plant ratios 1:1(E2A and TA), 1:10 (TA, Z3OH), 1:100 (TA), and 1:1000 (CIT) (Tukey´s test, P < 0.05). One compound (TA) also reduced the response to the second pheromone puff, and it was at the 1:1 ratio (Tukey´s test, P < 0.05). Plant blends differed in their effect on Z-ORN (df=2, F=9.08, P<0.001), where the Chinese blend produced significantly lower responses than the other two blends (Tukey´s test P<0.001). There was no significant interaction in *Z*-ORNs between plant doses and plant odorants (df = 70, F = 0.95, P = 0.6) or plant blends (df=14, F=0.81, P=0.65). Fig. 2C illustrates the response of a *Z*-ORN to pheromone, TA, and the blend of the two stimuli.

E-ORNs did not respond to hexane or the plant blends, and their response to *E*8-12:Ac was not affected by the addition of the plant blends (Fig. 5A and C, df=7, F=94.18, P<0.001).

4. Discussion

4.1 Plant-specific ORNs

In a previous study, we found that 29 % of the sensilla trichodea of male *G. molesta* contained ORNs that did not respond to the female sex pheromone components (Ammagarahalli and Gemeno, 2014). The present study reveals that

some of these pheromone-unresponsive ORNs may be tuned to plant-odorants. In general, the response of sensilla trichodea and sensilla auricillica ORNs to plant volatiles was not very different from the response to solvent stimulation, and a large percentage (probably more than 50 %, depending on the threshold criteria used) of these ORNs could be considered unresponsive to the stimuli panel that we have tested. Other moth studies report similar percentages of unresponsive ORNs in either sensilla trichodea [e.g., 71 % in *Trichoplusia ni* (Hübner) (Todd and Baker, 1993), 51 % in *S. littoralis* (Jönsson and Anderson, 1999), 22 % in *Manduca sexta* (L.) (Shields and Hildebrand, 2001)], or sensilla auricillica [60 % in *Cydia pomonella* (L.) (Ansebo et al., 2005)], so it appears that non-responding ORNs are not uncommon, but the reasons for such widespread ORNs "silence" are still unclear.

One possible explanation for the high number of unresponsive ORNs is that many plant ORNs have narrow molecular receptive ranges (MRR) (i.e., they are specialist), and that if the odour panel with which they are stimulated is modest (like the one we have tested), then only a few of them will show specific responses. Specialist plant ORNs are, indeed, common in moths (De Bruyne and Baker, 2008; Andersson et al., 2015), and in some cases they appear to be relatively abundant, such as the 30 % responding to phenyl acetaldehyde in *S. littoralis* (Binyameen et al., 2012). ORNs in moths are often categorized in response "types" according to their MRR (e.g., Shields and Hildebrand, 2001; Hillier et al., 2006; Binyameen et al., 2012), however in *G. molesta* the only specialist ORN "type" that we could identify was that responding to racemic farnesene (FAR) in sensilla trichodea. Further specialist responses were found in one or two ORNs, so they were not categorized as types. We explored the presence of further ORN types with statistical group analysis but it did not reveal more distinct ORN types than the already identified FAR

ORNs (data not shown). Comparison of dose-response curves between ph-ORNs (Ammagarahalli and Gemeno, 2014) and plant-ORNs (this study) in *G. molesta* reveals that plant-ORNs are at least one order of magnitude less sensitive than pheromone ORNs. To characterize plant ORNs we used a single plant dose of 100 ng, which is within the range of the dose-response curves (1 ng-10 µg). Higher plant doses may have resulted in stronger or more numerous ORN responses, but possibly at the expense of getting a distorted picture if the doses fall outside of the natural-response range (Hallem and Carlson, 2006). In *H. virescens* males the sensitivity of pheromone and plant-ORNs appears to be similar to each other (Hillier and Vickers, 2007), but in *A. ipsilon* males the pheromone ORNs are clearly more sensitive than the heptanal-responding ORNs (Barrozo et al., 2011).

The four FAR-specific ORNs that we describe in here were found only in sensilla trichodea, which suggest that there may be odour-specialization according to sensillum type, as has been observed in other species (Binyameen et al., 2012; Pophof et al., 2005). Interestingly, in two of the FAR ORNs there was a general reduction in spike frequency to most of the other odorants. To some extent this was also observed in the other two FAR ORNs, but we lost contact with them before testing the complete odour panel. Enhanced contrast due to a combination of excitation and inhibition was occasionally observed in other ORNs, mainly in sensilla auricillica. In addition, some of the individual compound responses were inhibitory instead of excitatory. Inhibition, as opposed to excitation, of plant-ORNs is rarely reported in the moth literature that we have examined, and in the few cases where it is reported, it is far less frequent than excitation (Anderson et al, 1995; Pophof et al., 2005). A Concert of excitatory and inhibitory responses to different compounds by the same ORN may increase the coding capability of plant-ORNs and help insects

decode a diverse plant stimulus landscape using less ORNs than if only excitatory responses were produced (Bruce and Picket, 2011; Clifford and Riffell, 2013).

Several farnesene isomers occur naturally as constituents of aphid alarm pheromone and apple coating volatiles, among other sources (e.g., Bowers et al., 1977). (*E*)-β-farnesene is an attractant of the tortricid moths *C. pomonella* (Yan et al., 2003), *Lobesia botrana* (Denis & Schiffermüller) (Tasin et al., 2009) and *G. molesta* (Il`ichev et al., 2009; Lu et al., 2012), and it excites ORNs in sensilla auricillica of *C. pomonella* (Ansebo et al., 2005). We tested a racemic farnesene mixture, so we do not know which isomer, or isomers, the FAR ORNs of *G. molesta* are tuned to. Another compound, the pear ester, has been shown to attract male *G. molesta* in dual-choice olfactory tests (Molinari et al., 2010), but other studies suggest that it is not attractive to *G. molesta* males in the field or in the laboratory (personal observation, and Knight and Light, 2004). Correspondingly, the ORN responses to this compound were mild and unspecific. By contrast, the pear ester is important in the behaviour of *C. pomonella*, and a high proportion of the ORNs respond with high specificity to this compound (Ansebo et al., 2005). Another compound that produced relatively specific responses in *G. molesta* was methyl salicylate. This compound was found in volatile collections of fruits but it was not tested behaviourally with *G. molesta* (Lu et al., 2012). *Mamestra brassicae* (L.) has a very specific ORN for methyl salicylate (Ulland et al., 2008) and this compound attracts *C. pomonella* (El-Sayed et al., 2013); therefore, it could be a potential attractant of *G. molesta*.

Most of the plant volatiles tested were chosen because of their demonstrated behavioural or physiological activity in *G. molesta* males or females (Il`ichev et al., 2009; Lu et al., 2012; Piñero and Dorn, 2007). Therefore, we expected to find more ORN responses than we did. These compounds were identified in non-floral plant

parts, but at least 14 of them are also present in flower scents (Knudsen and Tollsten, 1993). Flower odors are probably not very relevant to *G. molesta* males because this species is not known to visit flowers. Possibly, if odours emitted by the natural adult food sources were tested, more ORN responses would be obtained. Surprisingly little is known about the food sources of adult *G. molesta*, although presumably they feed in sugary plant secretions, like the extrafloral nectaries from peach leaves (Atanassov and Shearer, 2005).

4.2 Effect of plant volatiles on pheromone-specific ORNs

We reported previously that the *Z*8-12:Ac and *E*8-12:Ac pheromone-ORNs of *G. molesta* are very specific and sensitive to their natural ligands (Ammagarahalli and Gemeno, 2014). Here we show that ph-ORNs are not sensitive to the plant volatiles. We only tested the highest plant dose (100 ng) with the assumption that if ph-ORNs did not respond to a high plant dose they would not respond to the lower doses either. However, this premise, which is based on the general observation that the ORNs MRR broadens with increased stimulus doses (Hallem and Carlson, 2006), remains to be tested in this particular case. Despite the apparent lack of response of ph-ORNs to plant volatiles, when pheromone and plant stimuli were co-applied, the response of ph-ORNs was lower than when a comparable dose of the pheromone ligand was tested alone. This effect was consistent in the case of *Z*-ORNs tested with plant odorants, but sporadic or absent in the case of *Z*- and *E*-ORNs tested with plant odour blends.

Several characteristics of the pheromone-plant interaction at the ORN level prompt us to speculate that it would have only minor effects on male *G. molesta*

behaviour or ecology. First of all, although statistically significant, the reduction in spike frequency was only a modest 15 %, which, according to a calibrated response of ph-ORNs to pheromone doses (Ammagarahalli and Gemeno, 2014), is equivalent to stimulating the ORN with half the pheromone dose, and this would have only a minor effect on male behavioural response, since the number of males that contact an optimal pheromone source is stable over a 100-fold concentration step (Valera et al., 2011a). Secondly, there was no plant-dose effect, i.e., all pheromone:plant blends, from 1:1 to 1:1000, caused similar spiking activity reduction in *Z*-ORNs, which suggests that the suppression is somewhat independent of the plant volatiles themselves and could be due to other causes. For example, a mixture of mineral oil and hexane solutions can affect the release rate of compounds dissolved in each other (Ochieng et al., 2002). Nevertheless, the doses used in our experiment are probably in the same range than what a moth would encounter under natural conditions, as shown by the dose-response curves in the plant-specific ORNs. Furthermore, the effect of the plant volatiles was not cumulative because ph-ORNs responded similarly to the first sex pheromone puff as to the one following all the pheromone-plant stimulations, indicating that plant stimuli were not causing adaptation. Thirdly, although some plant odorants were slightly more active than others, their effect was fundamentally similar to each other, with a moderate reduction in ORN activity and no marked differences that could allow, *a priori*, sensory discrimination among them by the ph-ORNs. In brief, a modest dose-independent and unspecific decrease in ph-ORN firing rate by several plant odorants, may not, in itself, fully explain the pheromone-plant behavioural synergism that we have previously documented (Varela et al., 2011a).

In other moth species, the effect of plant volatiles on the response of ph-ORNs to pheromone is far more acute than what we report in here. In *A. ipsilon*, a 1:100 blend of pheromone:heptanal reduced responses from about 50 spikes s^{-1} with pheromone alone to about 5 spikes s^{-1} with the pheromone-plant blend, a level comparable to solvent stimulation (Deisig et al., 2012). In *H. virescens*, a 1:1 ratio of pheromone: linalool halved the spiking activity of *Z*11-16:Ac and *Z*11-16:Ald pheromone ORNs (Hillier and Vickers, 2011). In *H. zea*, 1:1 to 1:1000 ratios of *Z*11-16:Ald: linalool almost doubled spike frequency with respect to stimulation with pheromone alone (Ochieng et al., 2002). Furthermore, unlike *G. molesta*, in all these species the effect of the plant volatiles is dose-dependent. It is intriguing, though, that with few exceptions (Ochieng et al., 2002; Hillier and Vickers, 2011), in the majority of species where it has been tested, including *G. molesta*, the effect of plant volatiles is to decrease (and not increase) the response of ph-ORNs to pheromone (Deisig et al., 2014). This is counterintuitive because if pheromone-plant stimuli integration at the ph-ORN level were to explain behavioural synergism, one would expect that the mix would increase, and not decrease, ph-ORN responses to pheromone. A possible physiological function of pheromone suppression by plant volatiles is to improve pheromone pulse resolution, and thus potentially aid male orientation to pheromone-emitting females (Party et al., 2009; Deisig et al., 2014), although this remains to be tested with free-flying insects.

Yet, since plant-specific ORNs are already present on the moth antenna, it seems redundant that moths would also need to dedicate their highly-specialist ph-ORNs (De Bruyne and Baker, 2008) to sense plant volatile stimuli in order to gain additional information about the presence of plant volatiles in the environment. In addition, the available evidence indicates that pheromone and plant stimuli travel *via*

separate nerve lines to the AL and that integration takes place in there (Christensen and Hildebrand, 2002; Lei and Vickers, 2008; Namiki et al., 2008), so sensory integration at the peripheral level seems even more redundant. However, in the tortricids *C. pomonella* and *G. molesta* some projection neurons responding to pheromone innervate ordinary glomeruli and not the MGC located at the entrance of the antennal nerve, which typically receives pheromone input from the antenna (Trona et al., 2010, Varela et al., 2011b). This unusual pattern of coding in the AL could be explained by the response of non-pheromone ORNs to both pheromone and plant compounds at the peripheral receptor level in *C. pomonella* (Ansebo et al., 2005), or even in *G. molesta*, as we have shown. The accumulation of cases showing that plant volatiles, or even pheromone compounds (Hillier and Vickers, 2011), modify the response of ph-ORNs to cognate ligands (Deisig et al., 2014) deserves further study if we want to understand the possible ecological and behavioural functions of this physiological process.

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Appendix A. Supplementary material

S1) Ammagarahalli.suppl.tables (Supplementary tables)

S2) Ammagarahalli.suppl.data1 (raw data of ph-ORNs, used in statistical analysis, available as .txt file)

S3) Ammagarahalli.suppl.data2 (raw data of non-ph-ORNs, used in making raster plot, available as .xlsx file)

S4) Ammagarahalli.suppl.R.codes (R codes, output, and explanations)

Supplementary material associated with this article can be found, in the online version, at http://dx.doi.org/...

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Figure captions

Fig.1. ORN responses to plant volatiles. Sensilla contained between 2 and 3 ORNs characterized by their spike amplitude (L = large, M = medium, S = small). Response intensity is colour-coded according to the accompanying scale bar (spikes s^{-1}, relative to spontaneous activity). Lines group odorants of similar chemical type (*n*-hexane, pheromone, acetates, alcohols, aldehydes, aromatics, esters and terpenoids). A) 64 ORNs from 25 sensilla trichodea were tested with 3 to 10 odorants plus *n*-hexane and the pheromone blend. Maximum responses were -36 and +79 spikes s^{-1}. B) 88 ORNs from 40 sensilla auricillica were tested with 5 to 10 odorants, *n*-hexane and the pheromone blend, and 60 ORNs from 20 sensilla were tested with 19 to 20 odorants, *n*-hexane , the pheromone blend (PHE) and its individual compounds. Maximum responses were -35 and +97 spikes s^{-1}. Most cell responses were very similar to the response elicited by *n*-hexane (yellow range). Little specialization was observed, the most prominent being for racemic farnesene (FAR) occurring in the smaller ORNs of 4 sensilla trichodea (3S, 8S, 9S,18S). In sensilla auricillica, specific odorant responses were observed individual cells (e.g., 52M to 3OH, 32M to BZN, 52L and 53L to MS).

Fig.2. Illustrative SSR traces. A) Sensillum trichodeum housing 2 non-pheromone specialist ORNs, where the response of the smaller amplitude cell to racemic farnesene (FAR) is dose dependent, whereas the larger amplitude cell is unresponsive to this compound. B) Sensillum auricillicum housing 3 ORNs, the large amplitude ORN (left) responds to methyl salicylate (MS) whereas the medium

amplitude ORN (middle) responds to 1-octan-3-ol (3OH). A 100 ms trace on the right shows the three ORNs sizes (large [L], medium [M], and small [S]). C) A *Z*8-12:Ac-specific ORN in a sensillum trichodeum is not excited by terpinyl acetate (TA) (left) but its response to *Z*8-12:Ac (middle) is changed by TA (right). Numbers between parentheses correspond to cells shown in Fig. 1. The horizontal bar above each trace represents stimulus duration (200 ms).

Fig. 3. Dose-response curves of non-pheromone-specific ORNs housed in sensilla trichodea (A) and auricillica (B-F) of *G. molesta* males. Dotted lines are the ORNs response to *n*-hexane. Numbers correspond with ORNs shown in Fig. 1.

Fig. 4. Effect of plant odorants on the response of *Z*8-12:Ac ORNs to sex pheromone. ORNs (n = 8) were stimulated with *Z*8-12:Ac alone at 0.1 ng, *Z*8-12:Ac mixed with the plant odorant in 1:1 to 1:1000 pheromone: plant-odorant ratios (*Z*8-12:Ac at 0.1 ng), and a second 0.1 ng puff of *Z*8-12:Ac. A) Average response for each plant odorant and dose combination. B) *Z*8-12:Ac ORNs were not responsive to *n*-hexane or the plant odorants, but their response to *Z*8-12:Ac decreased when it was mixed with the plant odorant at all the doses. C) Some odorants caused more inhibition than others. Means in C are lower than in A and B because *n*-hexane and plant odorants are pooled in C. Different letters in B and C indicate significant differences among treatment means (Tukey pair-wise comparison after GLM, $P < 0.05$).

Fig. 5. Effect of plant odors (i.e., blends) on the response of pheromone-specific ORNs to sex pheromone and pheromone-plant blends. *Z*-ORNs (n = 8) and *E*-ORNs (n = 5) were stimulated with *n*-hexane, one of 3 plant blends (Australian, Chinese and Swiss, 100 ng), the pheromone ligand alone (*Z*8-12:Ac or *E*8-12:Ac ,0.1 ng, 1:0a), the pheromone ligand mixed with the plant odorant in 1:1 to 1:1000 pheromone: plant-odor ratios (pheromone at 0.1 ng), and a second 0.1 ng puff of the pheromone ligand (1:0b). A) Average response for each plant odor and dose combination. B) The response of *Z*-ORNs to *Z*8-12:Ac was reduced by the plant odors at pheromone:plant doses of 1:1 and 1:000. C) The response of *E*-ORNs to *E*8-12:Ac was not affected by the plant blends. Different letters indicate significant differences among treatment means (Tukey pairwise comparison after GLM, $P < 0.05$).

Table 1. Plant blends and individual plant odorants used in the study. Numbers indicate proportion of compounds in each plant blend. Abbreviation is provided for those compounds that were tested individually. * Found in volatile collections of apple and peach, but are not tested behaviourally. References provide behavioural relevance for each compound.

Plant compound	Blend composition			Abbreviation	References
	Chinese	Swiss	Australian		
1-hexanol	1				Lu et al., 2012
nonanal	1			NON	Lu et al., 2012
ethyl butanoate	100			EB	Lu et al., 2012
butyl acetate	70				Lu et al., 2012
ethyl hexanoate	7			EH	Lu et al., 2012
hexyl acetate	5			HA	Lu et al., 2012
hexyl butanoate	1			HB	Lu et al., 2012
farnesene (racemic)	4		100	FAR	Lu et al., 2012; Il'ichev et al., 2009
ocimene (racemic)			100		Il'ichev et al., 2009
(*Z*)-3-hexenyl acetate		100	50	Z3HA	Piñero and Dorn, 2007; Il'ichev et al., 2009
(*Z*)-3-hexenol		20		Z3OH	Piñero and Dorn, 2007
(*E*)-2-hexenal		3		E2A	Piñero and Dorn, 2007
benzaldehyde		20		BZA	Piñero and Dorn, 2007
benzonitrile		0.5		BZN	Piñero and Dorn, 2007
pear ester (ethyl (*E*,*Z*)-2,4-decadienoate)				PE	Knight et al., 2014
citral				CIT	Faraone et al., 2013
3-methylbutyl acetate				3MBA	Lu et al., 2012
terpinyl acetate				TA	Knight et al., 2014
(*E*)-β-caryophyllene				CAR*	Natale et al., 2003
butyl hexanoate				BHX	Lu et al., 2012; Natale et al., 2004
butyl butanoate				BBT*	Lu et al., 2012
octyl acetate				OA*	Wang et al., 2009
methyl salicylate				MS*	Lu et al., 2012
1-octen-3-ol				3OH*	Casado et al., 2006

Fig. 1

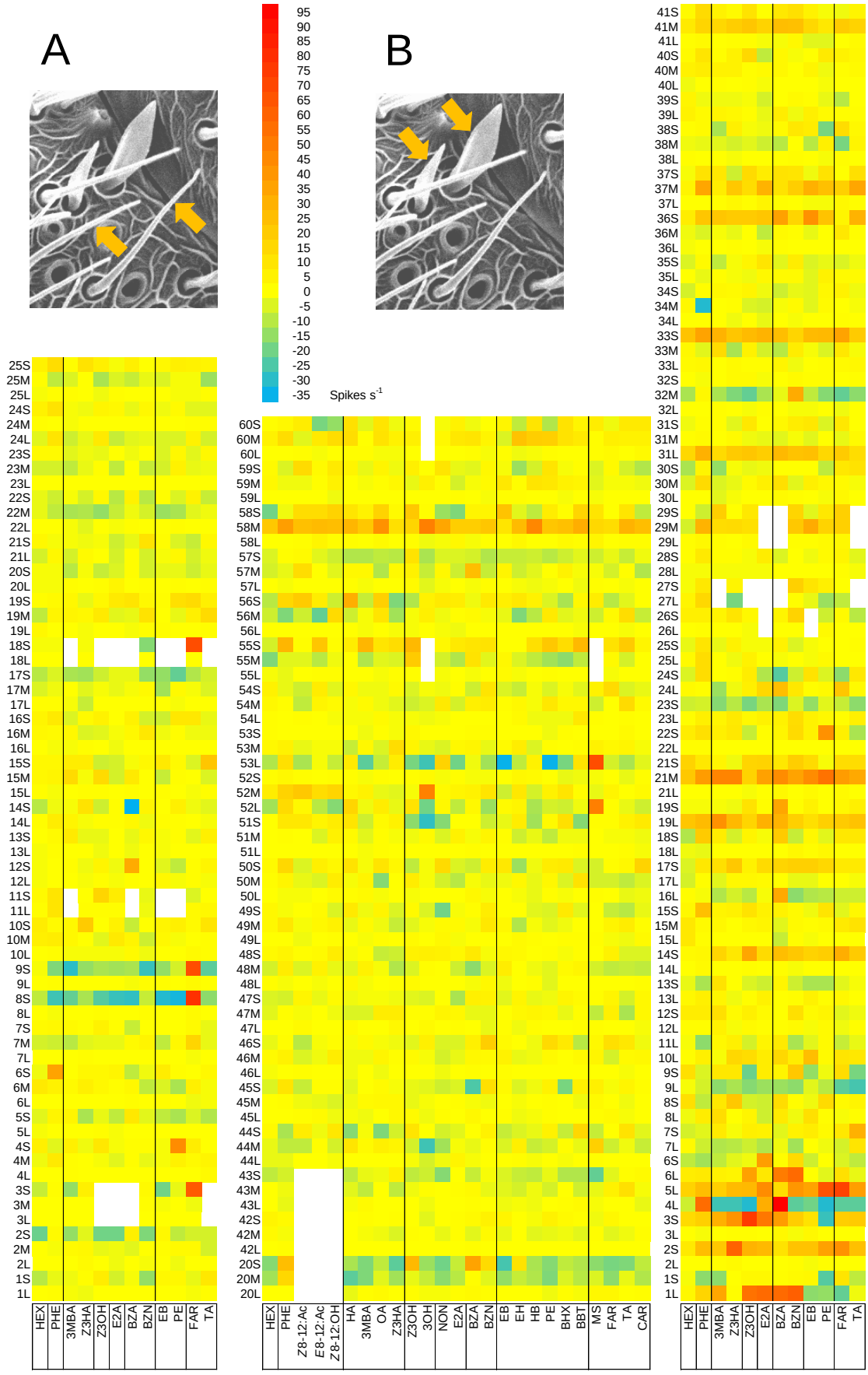

A
B
95
90
85
80
75
70
65
60
55
50
45
40
35
30
25
20
15
10
5
0
-5
-10
-15
-20
-25
-30
-35
Spikes s-1
HEX PHE 3MBA Z3HA Z3OH E2A BZA BZN EB PE FAR TA
HEX PHE Z8-12:Ac E8-12:Ac Z8-12:OH HA 3MBA OA Z3HA Z3OH 3OH NON E2A BZA BZN EB EH HB PE BHX BBT MS FAR TA CAR
HEX PHE 3MBA Z3HA Z3OH E2A BZA BZN EB PE FAR TA

Figure 2

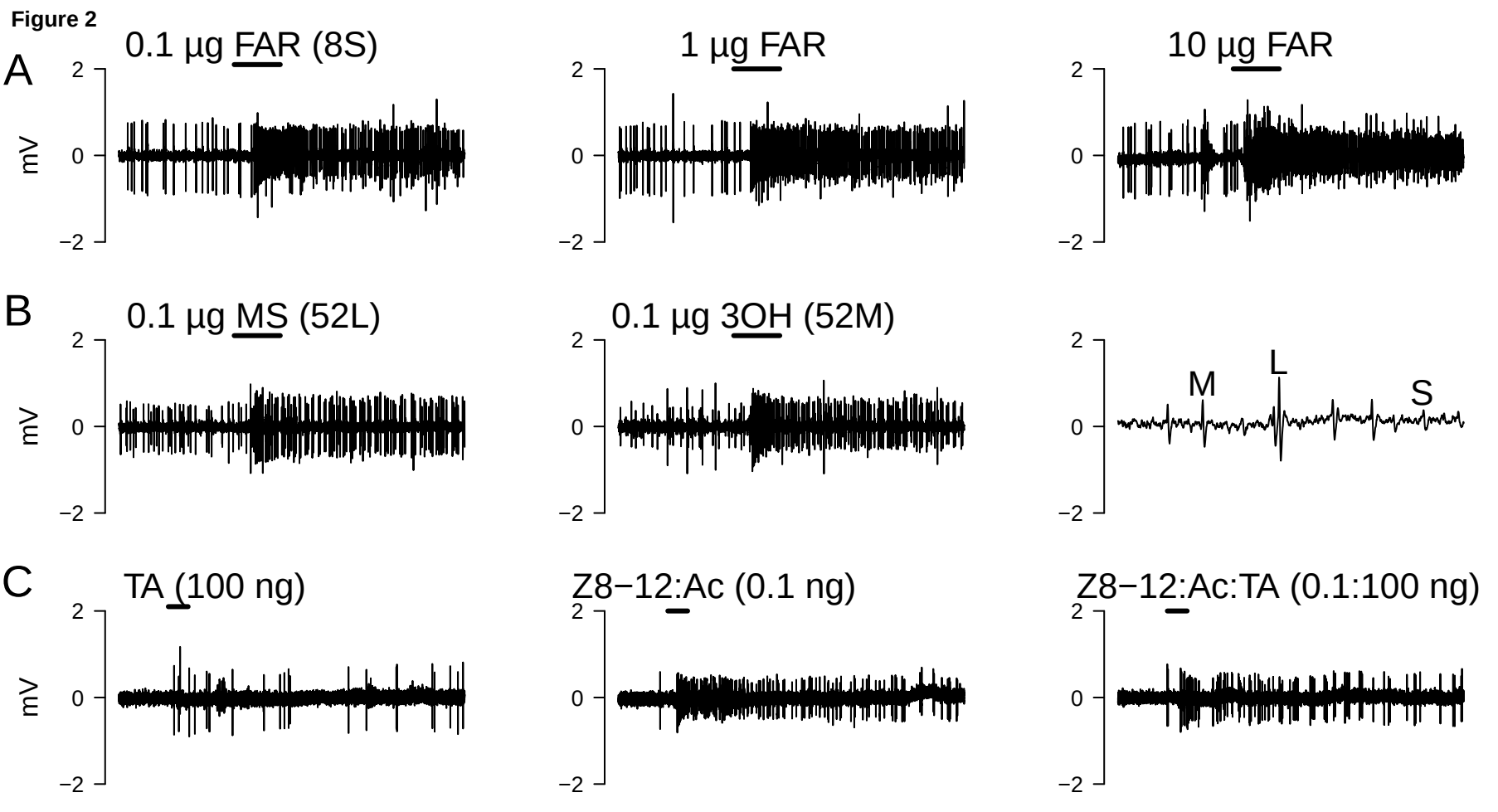

A
0.1 µg FAR (8S)
1 µg FAR
10 µg FAR
B
0.1 µg MS (52L)
0.1 µg 3OH (52M)
M
L
S
C
TA (100 ng)
Z8−12:Ac (0.1 ng)
Z8−12:Ac:TA (0.1:100 ng)
mV
2
0
−2

Figure 3

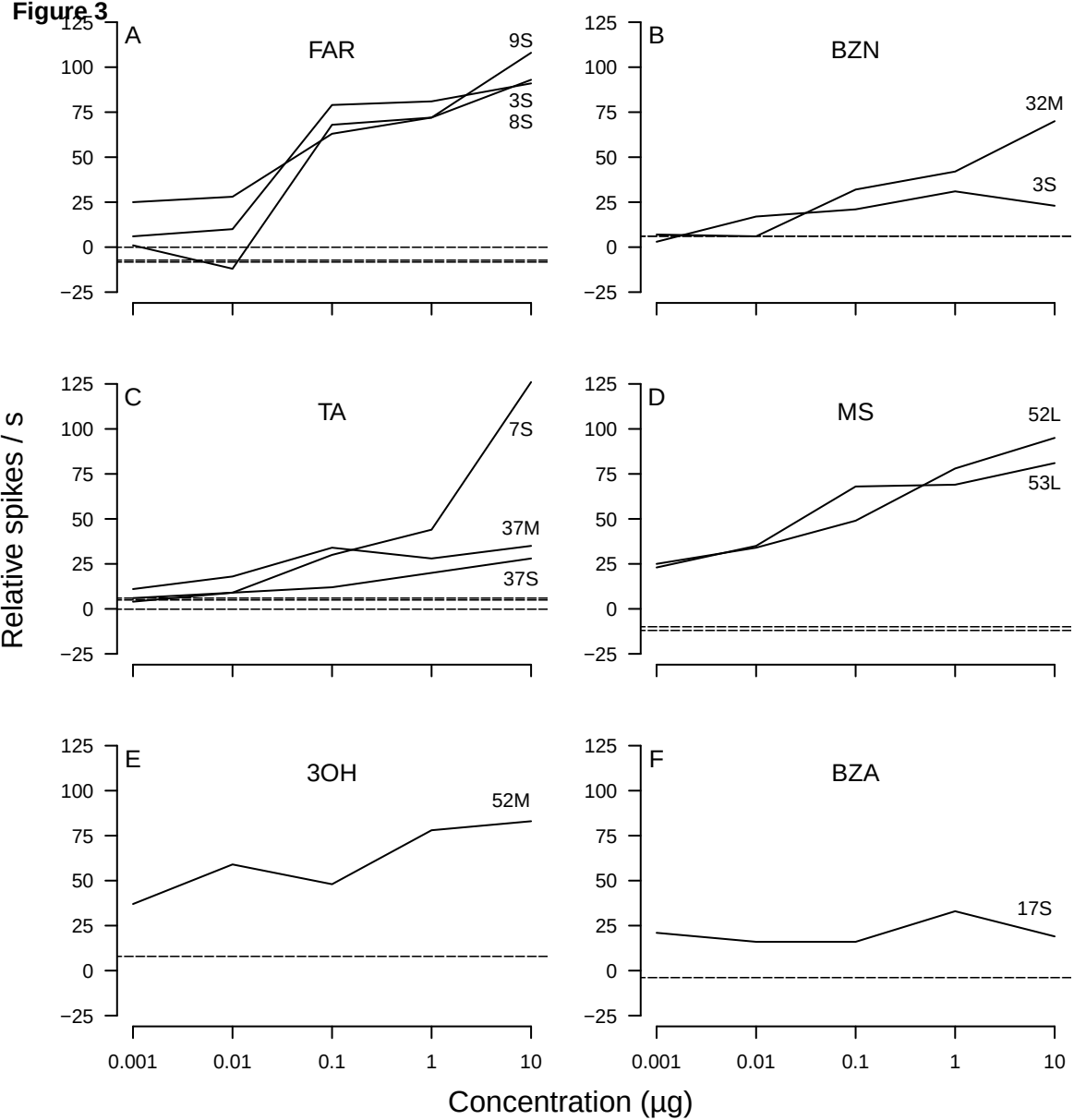

A
FAR
9S
3S
8S
B
BZN
32M
3S
C
TA
7S
37M
37S
D
MS
52L
53L
E
3OH
52M
F
BZA
17S
Relative spikes / s
125
100
75
50
25
0
−25
0.001
0.01
0.1
1
10
Concentration (µg)

Figure 4

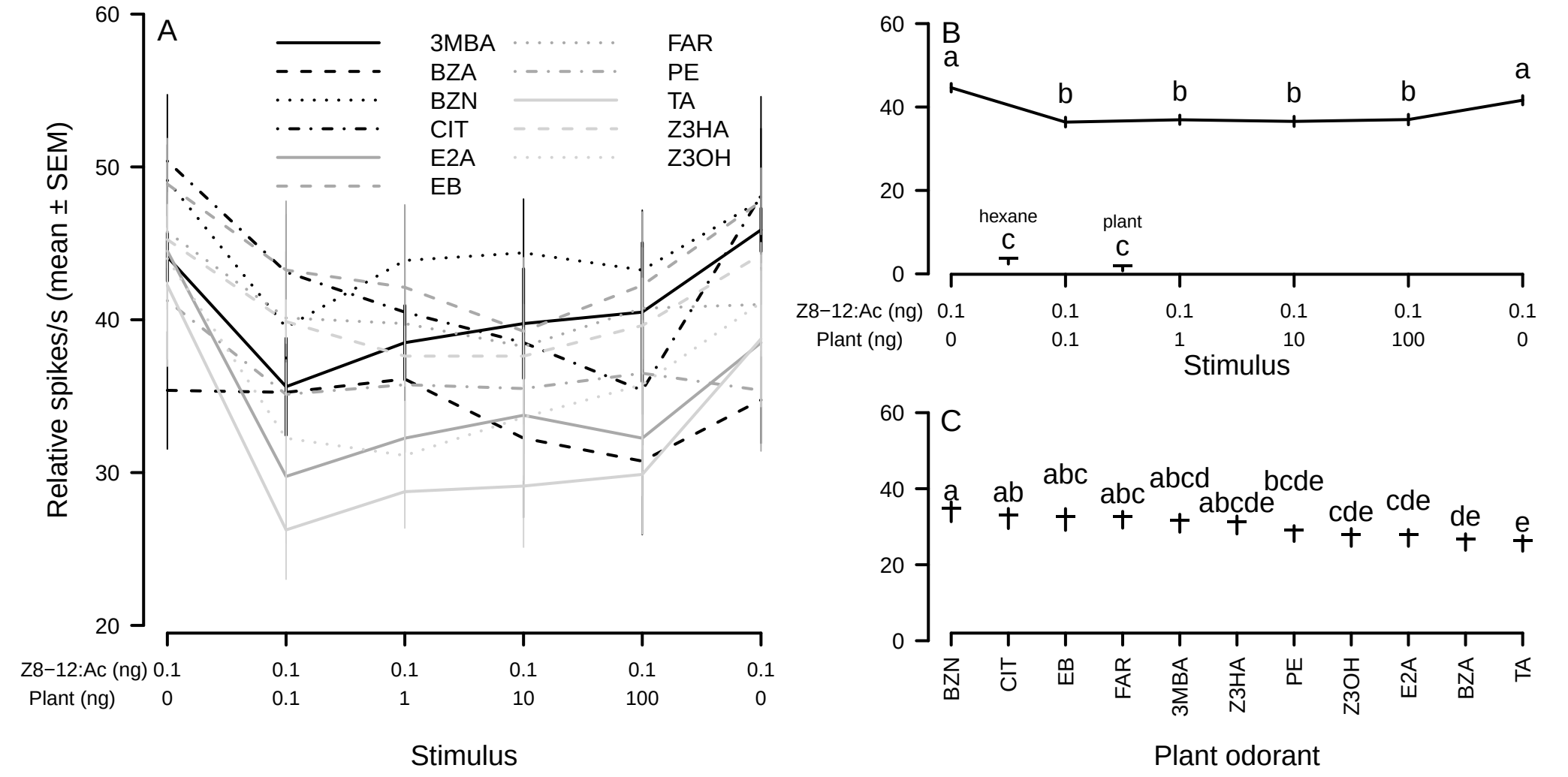
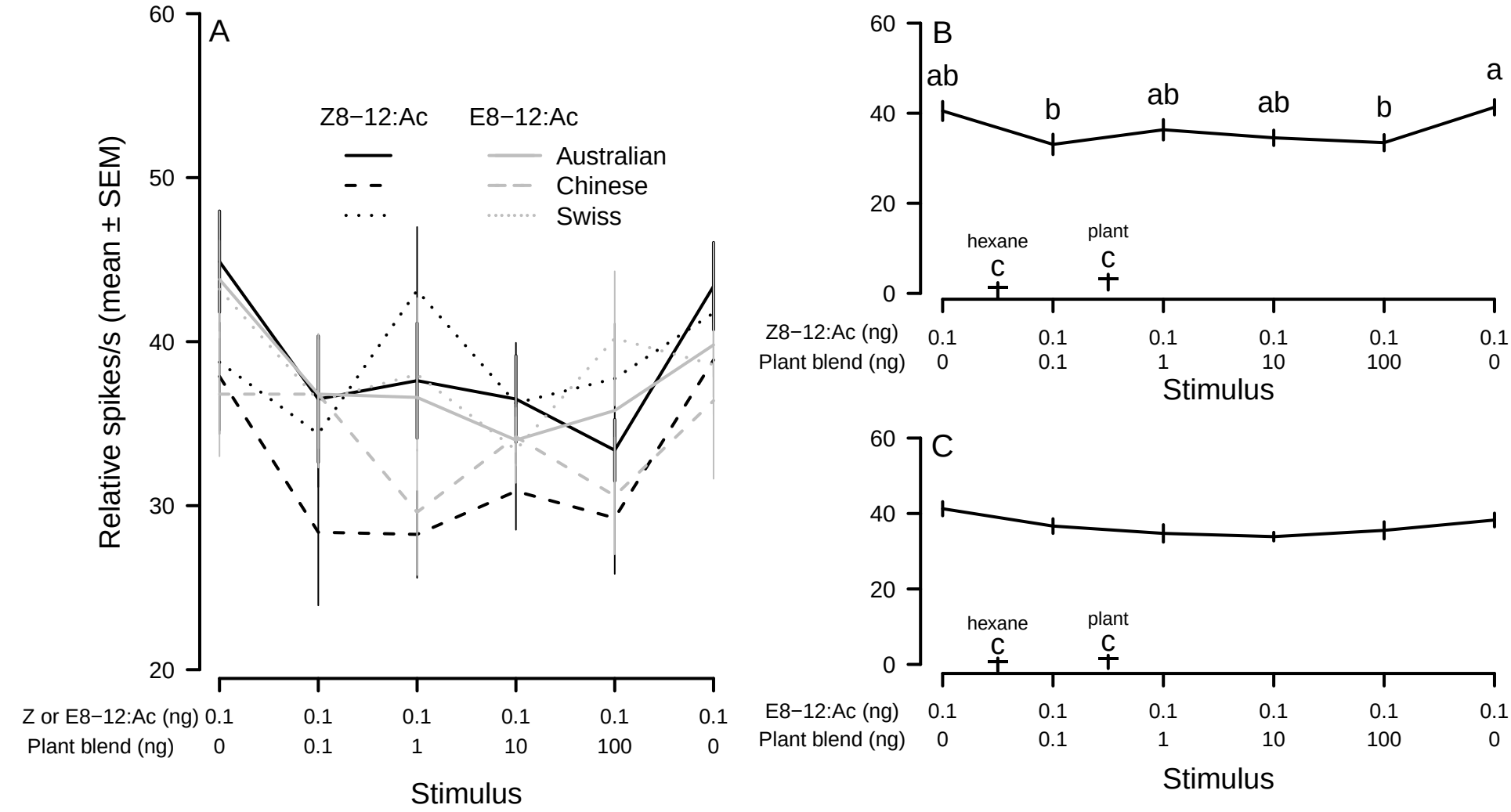

A
3MBA
BZA
BZN
CIT
E2A
EB
FAR
PE
TA
Z3HA
Z3OH
Relative spikes/s (mean ± SEM)
60
50
40
30
20
Z8−12:Ac (ng) 0.1 0.1 0.1 0.1 0.1 0.1
Plant (ng) 0 0.1 1 10 100 0
Stimulus
B
a b b b b a
hexane
plant
c c
60
40
20
0
Z8−12:Ac (ng) 0.1 0.1 0.1 0.1 0.1 0.1
Plant (ng) 0 0.1 1 10 100 0
Stimulus
C
a ab abc abc abcd abcde bcde cde cde de e
60
40
20
0
BZN CIT EB FAR 3MBA Z3HA PE Z3OH E2A BZA TA
Plant odorant

Figure 5


A
Relative spikes/s (mean ± SEM)
60
50
40
30
20
Z8−12:Ac
E8−12:Ac
Australian
Chinese
Swiss
Z or E8−12:Ac (ng) 0.1 0.1 0.1 0.1 0.1 0.1
Plant blend (ng) 0 0.1 1 10 100 0
Stimulus
B
ab b ab ab b a
hexane plant
c c
Z8−12:Ac (ng) 0.1 0.1 0.1 0.1 0.1 0.1
Plant blend (ng) 0 0.1 1 10 100 0
Stimulus
C
hexane plant
c c
E8−12:Ac (ng) 0.1 0.1 0.1 0.1 0.1 0.1
Plant blend (ng) 0 0.1 1 10 100 0
Stimulus